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REMARKS

A. Regarding the Amendments

Claims 1, 9, and 15 have been amended. Claims 1, 9, and 15 are amended to recite to administration of a bolus injection. The amendment is made to clarify the claimed subject matter. The amendment is supported in the specification at, for example, page 21, lines 15-17, and at, for example, page 22, lines 11-16.

Claims 9 and 15 are also amended to recite to a method for reducing neurological inflammation. The amendment is made to clarify the claimed subject matter by providing proper basis in the preamble for the term "reducing neurological inflammation." The amendment is supported in the specification at, for example, page 33, line 18, to page 35, line 6 (see also Figures 6 and 7 and Table 3). New claim 22 has been added to further define the invention.

Therefore, the claim amendments do not introduce new matter. Upon entry of the current amendment, claims 1-2, 4-5, 7, 9-10, 12-13, 15-16, 19 and 22 will be pending.

B. Status of Application

It is stated that the amendment made December 5, 2003 has been entered, and that claims 1-2, 4-5, 7, 9-10, 12-13, 15-16, and 19 are under consideration.

C. Withdrawn Objections/Rejections

Applicants gratefully acknowledge withdrawal of the rejection (made in the Office Action mailed June 30, 2003) of claims 1-16 and 19-21 pursuant to 35 U.S.C. § 112, first paragraph. Applicants also gratefully acknowledge withdrawal of the rejection of claims 1-16 and 19-21 under 35 U.S.C. § 112, second paragraph, made in the same Office Action.

D. Rejection Under 35 U.S.C. § 112, Second Paragraph (Definiteness)

The rejection of claims 9, 12-13, 16, and 19 under 35 U.S.C. § 112, second paragraph, as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicants regard as the invention, is respectfully traversed as moot in light of the claim amendments. Specifically, Applicants note that claim 9 is amended to specify a method for reducing neurological inflammation rather than reducing inflammation in general. Claims 12-13, 16, and 19, which depend from claim 9, will accordingly meet the requirement of 35 U.S.C. §112, second paragraph in light of the amendment to claim 9.

Therefore, Applicants submit that the rejection under 35 U.S.C. § 112, second paragraph is moot and respectfully request that the rejection be withdrawn.

E. Rejection Under 35 U.S.C. § 102(e)

The rejection of claims 1-2, 4, 9-10, 12, and 15 under 35 U.S.C. § 102(e) as anticipated by Grinnell (U.S. Patent No. 6,268,337) is respectfully traversed. It is alleged in the Office Action that the '337 patent teaches intravenous administration of activated protein C ("APC") to subjects with vascular occlusive and arterial thromboembolytic disorders (citing columns 3-4 and column 8, lines 28-33).

Grinnell '337 describes intravenous administration of APC to a group of twelve dogs (Example 2). According to the '337 patent, a segment of the dogs' left circumflex **coronary artery** ("LCCA") was isolated and instrumented with an electromagnetic flow probe (to measure coronary blood flow), a stimulating electrode (to produce vessel injury), and an external occluder (to produce critical stenosis). Total vessel occlusion was achieved in about 60 minutes. The dogs were administered a **continuous intravenous infusion** of 2.0 mg/kg/hr APC for two hours, commencing 30 minutes after total vessel occlusion occurred.

In contrast, claim 1 teaches a method of using APC to protect neuronal cells from cell death, thereby reducing neurological damage. Claim 1 as currently amended describes the administration of a **bolus injection** containing an effective amount of APC, which can be carried out, for example, within six hours of a stroke (e.g., see claim 4). As disclosed in Example 1, mice treated with APC before and after the middle **cerebral artery** was occluded exhibited a 25% increase in cerebral blood flow ("CBF") as compared with a control group. APC did not affect CBF in the non-ischemic hemisphere (Figure 1 and Table 1). During reperfusion, CBF increased to between 71-98% of baseline levels in APC-treated mice, which approaches pre-ischemic CBF values (see Figure 1B). The mean survival time of control mice was 10.2 hours, as contrasted with 23.7 hours for APC-treated mice (Figure 2 and Table 2). APC treatment also resulted in a significant decrease in the volume of brain injury.

In addition, APC treatment resulted in a significant decrease of fibrin-positive microvessels in the ischemic hemisphere in the area of infarction (Figure 4A), a decrease in polymorphonuclear leukocytes (PMNs, Figure 4B), and an absence of microbleeding in ischemic brain tissue (Figure 4C). These results were corroborated by Western blot analyses (Figure 5), which indicates an 8.2-fold decrease of fibrin deposited in tissue (in the area of infarction) when APC-treated mice were compared with control mice. Fibrin is a protein that forms at wound sites, so this data shows that APC treatment reduced neurological inflammation and damage. The decrease of PMNs indicates that APC prevents intravascular accumulation of PMNs in the brain by preventing their transport across the blood-brain barrier (see the specification at, for example, page 9).

The results shown in Example 2, which was essentially carried out using the method of Example 1, further support the finding that APC provides neuroprotective benefits. In Example 2, APC (2 mg/kg) was administered to mice 10 minutes after MCA occlusion (see also page 24, line 29, to page 25, line 2 for timing of administration of APC). This resulted

in a 69% decrease in infarction volume (Figure 6A), a 69% decrease in edema volume (Figure 6B), restoration of CBF to control levels (Figure 6C), and elimination of brain accumulation of neutrophils (Figure 6D). Survival scores 24 hours after MCA occlusion/ reperfusion are shown in Table 3. After 24 hrs., subjects were sacrificed in order to quantify brain injury. As shown in Figure 7, 0.1 mg/kg of APC was not sufficient to obtain the protective benefits shown at higher dose levels (see also the specification at page 33, lines 27-29, which describes the dose-dependency of APC treatment).

While Grinnell '337 shows the intravenous administration of APC, it does not teach the use of a **bolus injection** containing APC. In the rare instance that Grinnell describes the use of a bolus injection, it is in conjunction with continuous intravenous infusion of APC, however, it is important to reiterate that Grinnell does not describe reduction of neurological damage or alteration in cerebral blood flow. Grinnell describes the therapeutic use of APC when administered after coronary occlusions in dogs. However, Grinnell does not teach or suggest the neuroprotective benefits of APC treatment as disclosed by claim 1 and Example 1, which discloses neuroprotective data shown in mice. In addition, the dose levels and efficacy of APC treatment is species-specific (see the specification at, for example, page 20, lines 22-29). Therefore, Grinnell '337 does not anticipate claim 1, and withdrawal of this rejection is respectfully requested.

It is further submitted that Grinnell '337 does not anticipate independent claims 1, 9, and 15 (and their dependent claims), because the studies described in Grinnell could not provide the protective benefits of APC administration by bolus injection. Example 1 of Grinnell '337 describes the administration of APC in a group of six humans. As with Grinnell's dog study, continuous infusion of APC was performed. Plasma concentration of APC was measured using a clotting assay. Various dose levels were tested, and the conclusion was that the optimal dose for treatment of thrombotic stroke would be 0.024 mg/kg/hr.

It is also stated that the '337 patent shows that administration of APC is beneficial in preventing the local extension of the microvascular and macrovascular occluding arterial thrombus, thereby reducing the neurological deficit resulting from stroke (citing the abstract). As discussed above, the '337 patent describes coronary artery models and does not show neuroprotective results based on a cerebral artery model. Specifically, the procedures described in the '337 patent are designed to create and then dissolve coronary blood clots in the subject. The claimed invention is not based on dissipation of an existing or created blood clot. Rather, it is based on blocking cerebral blood flow.

It is further stated that the claimed invention says that those *at risk* of having stroke can be given APC, which could be interpreted as the entire human population. However, the phrase "at risk" could also be interpreted to mean a much smaller subset of the human population as specifically defined in the specification as filed (see page 15, lines 2-5, reproduced herein):

> In another embodiment, the present invention provides methods for protecting neuronal cells from cell death in a subject having or at risk of having a neuropathological disorder is provided. The method includes administering to the subject, a neuroprotective effective amount of activated protein C (APC), for example, in a pharmaceutically acceptable carrier, thereby providing neuroprotection to the subject. Examples of "neuropathological disorders" include but are not limited to stroke, Alzheimer's disease, Huntington disease, ischemia, epilepsy, amyotrophic lateral sclerosis, mental retardation and aging. One "having or at risk of having" an inflammatory vascular disease as described herein is a subject either exhibiting symptoms of the disease or diagnosed as being at risk for developing the disease. Such subjects include those subjects having undergone or preparing for surgical procedures as described below.

For example, the subset could include only individuals with a family history of stroke and/or individuals who have experienced one or more strokes.

Accordingly, Grinnell '337 does not teach each and every element of the pending claims and Applicants respectfully request withdrawal of the rejection under 35 U.S.C. §102.

F. Rejection under 35 U.S.C.§ 103(a)

The rejection of claims 1-2, 4-5, 7, 9-10, 12-13, 15 and 19 under 35 U.S.C. § 103(a) as allegedly unpatentable over Grinnell et al. (U.S. Patent No. 6,268,337) in view of Arnljots and Hickenbottom is respectfully traversed.

In order for an invention to be obvious, the differences between the subject matter of the application and the prior art must be such that the subject matter as a whole would have been obvious at the time the invention was made to a person of ordinary skill in the art. In order to meet this standard, the combination of references must teach or suggest all of the elements of the claimed invention. It is respectfully submitted that Grinnell et al. (U.S. Patent No. 6,268,337), even when taken in view of Arnljots and Hickenbottom, does not teach or suggest all of the elements of the claimed invention.

A showing of obviousness requires three elements: 1) there must me some suggestion or motivation to combine the reference teachings; 2) there must be a reasonable expectation of success; and 3) the combined prior art references must teach or suggest all of the claim limitations. It is respectfully submitted there was no suggestion or motivation to combine Grinnell et al. with Arnljots and/or Hickenbottom and that there would not have been any expectation of success in any such combination. It is also submitted that the references cited, when taken together, do not teach or suggest all of the elements of the claimed invention.

The teaching or suggestion to make the claimed combination and the reasonable expectation of success must be both found in the prior art and cannot be based on Applicant's disclosure. Section 2141.01 of the MPEP states that a rejection of claims under 35 U.S.C. § 103 must be made without the benefit of hindsight afforded by the claimed invention. It is respectfully submitted that the present rejection for obviousness is based on improper hindsight.

Grinnell '337 has been described in detail above. Arnljots describes the antithrombotic properties of treatment of APC with Protein S in rabbits. The subjects were given bolus injections of 0.1 mg/kg of bovine APC alone or combined with Protein S (0.5, 0.1, or 0.05 mg/kg). The injections were administered following arteriotomy and five minutes prior to reperfusion. Vessel patency (seems similar to blood flow level) rates were then assessed to determine antithrombotic response. Figure 1 shows vessel patency data, which was taken at 30 and 120 minutes following reperfusion. Arnljots describes the significance of protein S levels, the importance of the active site in APC in antithrombotic effect (active-site-blocked APC had no antithrombotic effect), as well as the importance of the structural integrity of the thrombin-sensitive region of protein S.

However, Arnljots does not describe the neuroprotective effects of APC treatment. The claimed invention shows the beneficial synergistic effects of administering APC with Protein S, a non-enzymatic cofactor of APC (see Example 3 and Figure 8). Also, as discussed above, APC treatment is highly species-specific. In particular, APC is shown to be species-specific for the Protein S cofactor (see the specification at, for example, page 24, lines 18-26). Finally, Arnljots teaches away from the claimed invention to a degree by suggesting the need to combine Protein S with APC in order to achieve positive results. In contrast, the claimed invention describes not only the benefits of APC administered concurrently with protein S, but the positive neurological effects of APC alone.

Hickenbottom describes various neuroprotective agents as well as the need to administer them within a few minutes prior to a few hours after the onset of ischemia. Hickenbottom further discusses the strategy of targeting various stages in the ischemic cascade, and goes on to describe therapeutic uses of hypothermia in treating head traumas, while noting that hypothermia has not been tested as a possible treatment for stroke. However, Hickenbottom does not describe the use of APC, either alone or in conjunction with Protein S, as a neuroprotective agent. It would not have been obvious, taking the

disclosure of the art cited of the effects of APC treatment on thromboembolism, in combination with Hickenbottom's discussion of possible neuroprotective agents in combating the negative effects of ischemia, to consider and then examine the possibility of using APC as a neuroprotective agent.

Accordingly, Applicants submit that inventive steps were required, which would not have been obvious to those skilled in the art, to obtain the claimed methods of using APC for neuroprotection. As the cited references do not suggest or motivate combination of their teachings, one of skill in the art would not have reasonably expected the combination of references to produce a method useful for neuroprotection. Additionally, because all of the cited references taken together do not teach or suggest all of the elements of the claimed invention, it is respectfully submitted that Grinnell et al. in view of Arnljots and Hickenbottom does not render the claimed invention obvious under 35 U.S.C. § 103(a). Therefore, withdrawal of this rejection is respectfully requested.

Claim 16 is also rejected under 35 U.S.C. § 103(a) as anticipated by Grinnell '337, Arnljots, and Hickenbottom as applied to claims 1-2, 4-5, 7, 9-10, 12-13, 15, and 19 in further view of Grinnell '514. Claim 16 depends from any of claims 1, 9, or 15, and further provides the administration of an anticoagulant, an anti-platelet, and/or a thrombolytic agent. For the reasons discussed above, claims 1, 9, and 15 are not obvious in view of Grinnell '337, Arnljots, and Hickentbottom. Similarly, the claims are not obvious when taken in further view of Grinnell '514.

Grinnell '514 describes the use of APC for treating thrombotic disorders in guinea pigs. In addition, as detailed above, APC treatment is highly species-specific. Grinnell does not teach the use of APC administered by bolus injection as a neuroprotective agent and for reduction of neurological damage following stroke. Therefore, withdrawal of this rejection is respectfully requested.

Finally, claims 15-16 and 19 are objected to as anticipated by Griffin (U.S. Patent No. 5,084,274)(our inventors are Griffin and Zlokovic) in view of Arnljots. It is stated that Griffin teaches the intravenous administration of APC to subjects with arterial thrombotic occlusion or thromboembolism (citing column 3, lines 8-19 and columns 5-8). It is further alleged that Griffin discloses that APC may be administered alone or in combination with a thrombolytic agent (citing column 2, lines 9-20).

Griffin (U.S. Patent No. 5,084,274) describes the use of APC, either alone or with a thrombolytic agent (such as tPA), to prevent arterial thrombotic occlusion. Baboons were used as test subjects, and given a combination of a bolus injection followed by continuous infusion. A variety of dose levels were used, ranging from 2.0 to 11 mg of APC (total), as well as a combination of 2.1 mg APC with 1.3 mg tPA. APTT assays were used to measure the plasma concentration of APC following treatment. Although Griffin notes the treatment can be applied to coronary, cerebral, or peripheral arteries, the data provided was from coronary arteries. In addition, the focus of the results described in Griffin was to prevent or dissipate arterial blockage. Griffin does not, however, describe the use of APC as a neuroprotective agent as disclosed in the claimed invention.

It is further alleged in the Office Action that the state of the art is such that arterial thrombotic occlusion and thromboembolism are inflammatory vascular diseases characterized by the closure of a blood vessel by a blood clot causing blockage of blood flow, and that an inflamed arterial segment has the tendency to embolize tiny particles of microparticulate atheromatous debris, platelet thrombi, or both, into the microvasulature. It is also stated that elevated levels of circulating inflammatory mediators in patients with ischemic heart disease have been reported (citing Yamada et al.).

Yamada describes inflammation, mainly with respect to heart disease. While Yamada notes levels of circulating inflammatory mediators in patients with heart disease,

this information would not motivate one skilled in the art to combine it with the other cited references to apply thrombolytic treatment to the brain. In addition, there would be no reasonable expectation of success in such a combination.

Several references provide data which suggests that applying the same thrombolytic treatment used for blockages in coronary vessels to blockages in cerebral vessels would not be preferable. For example, Traynelis (*Nature Medicine* 7(1): 17-18 (2001); Exhibit A) describes the use of tissue plasminogen activator (tPA) as a desirable agent for dissolving myocardial blood clots. However, Traynelis notes that while tPA may be effective for dissolving clots in the brain, tPA may also be toxic to brain cells (see also Grotta, *Circulation* 107:2769-2770 (2003), Exhibit B, which states that treatment of acute stroke with antithrombotic therapies alone has not been effective).

It is stated that Griffin does not teach the administration of a therapeutically effective amount of Protein S, but that Arnljots teaches that the anti-coagulant action of APC is enhanced by the nonenzymatic cofactor Protein S (citing page 937, paragraph 3). It is also stated that Arnljots teaches that, in considering the use of APC as an antithrombotic agent in humans, their findings suggest coadministration of Protein S to be beneficial (citing page 940, last paragraph).

It is alleged that it would have been obvious to a person skilled in the art to modify the APC method described by Griffin by coadministering Protein S as taught by Arnljots. It is further stated that there was a motivation to combine references, there was a reasonable expectation of success, and therefore the invention is *prima facie* obvious. It is also stated that the claimed invention covers the same patient population treated with the same compound as shown in Griffin et al., and therefore the same inherent results are produced. Again, it is noted that an entire population could be considered "at risk" of having stroke.

The Office Action goes on to quote *In re Papesch*, stating "A compound and all of its properties are inseparable; they are one and the same thing." The Examiner notes that simply stating a new property of APC does not render the method of reducing inflammation in a subject of the instant application free of art. Finally, it is stated that the claims do not recite to specific dosages of APC.

It is respectfully submitted that Applicants have done more than state a new property of APC. While a compound and its properties may be inseparable, numerous experiments using several different methods and combinations of agents were required to ascertain the neuroprotective properties of APC as opposed to its anti-coagulant properties.

In addition, as discussed in detail above, the prior art does not anticipate or obviate the claimed invention. Prior studies focused on dissipation of coronary blood clots by continuous infusion of APC, and used a variety of animal subjects such as dogs and baboons. The instant invention, in contrast, discloses a method that protects against impending neurological damage in cerebral vessels by administration of a bolus injection (or series of bolus injections) of APC in mice and humans. Further, the claimed invention discloses the anti-coagulant and anti-inflammatory effects of APC in the brain.

Current studies continue to attempt to understand the complex mechanisms of APC. For example, Cheng et al. (Nature Medicine 9(3): 338-342, 2003) sought to determine whether APC's neuroprotective effects are secondary to its anti-coagulant and anti-inflammatory effects *in vivo*. Cheng et al. found that low dose levels of mouse APC had neuroprotective effects independent of its anti-coagulant effects. Cheng et al. tested hypoxic and ischemic brain endothelial cells (BECs) with different agents. The agents used were: recombinant mutant APC (lacking the active-site serine), protein C zymogen, heat-inactivated APC, and APC with neutralizing C3 monoclonal antibodies against APC (see

Figure 1e). The only agent found to provide direct cytoprotection was APC, indicating that the active-site serine of APC is necessary to obtain the cytoprotective effects of APC.

Cheng et al. also undertook analysis of whether the *in vivo* mechanisms of APC involve anti-coagulant and anti-inflammatory pathways. Both low dose (0.04 mg/kg) and high dose (0.2 mg/kg) APC were administered. While low dose APC reduced motor neurological score and volume of brain injury (by 73% and 38%, respectively), high dose APC resulted in neuroprotective benefits of an even greater magnitude (see Figures 3e and 3f). High dose APC resulted in a 91% reduction in motor neurological score, a 65% reduction in volume of brain injury, a 30% improvement in post-ischemic CBF, a notable reduction in fibrin deposition, and a moderate reduction in CD11b (neutrophil) deposition (see Figures 3e through 3i). Based on these results, Cheng et al. theorized that neuroprotective activity of APC is distinct from its anti-coagulant activity *in vivo*.

The present invention exceeds the findings of Cheng et al. by providing empirical data showing that APC is directly neuroprotective. Recent data supplements this finding (see Guo et al., Neuron 41: 563-572, 2004; Exhibit C). Guo et al. show that APC directly protects mouse neurons in culture from N-methyl-D-aspartate ("NMDA"), an inducer of neuronal apoptosis (see the Results section and Figures 1 to 5). In addition, APC is shown to protect mouse neurons from staurosporine, another inducer of neuronal apoptosis (see the Results section and Figures 6 and 7). These results demonstrate the direct neuroprotective effects of APC exclusive of its anti-coagulant or other effects, since an *in vitro* method was utilized. In contrast, the art cited by the Examiner specifically relies upon the anti-coagulant effects of APC. Guo et al. also provide information on the cellular pathways of APC and the significance of PAR1 and PAR3 receptors with respect to APC neuroprotective activity (see page 566 and Figure 5D). These findings provide additional support for the disclosure of the claimed invention.

Therefore, it is respectfully submitted that the prior art does not anticipate or obviate the claimed invention. Applicants have done more than simply state a new property of APC. Inventive steps were required to discover the unknown neuroprotective activity of APC. The instant invention discloses in detail a method that protects against impending neurological damage in cerebral vessels by administration of APC. Accordingly, removal of this rejection is respectfully requested.

CONCLUSION

In view of the above amendments and remarks, reconsideration and favorable action on claims 1, 2, 4, 5, 7, 9, 10, 12, 13, 15, 16, 19 and 22 is respectfully requested. In the event any matters remain to be resolved, the Examiner is requested to contact the undersigned at the telephone number given below so that a prompt disposition of this application can be achieved.

Respectfully submitted,

Date: July 23, 2004

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Is tissue plasminogen activator a threat to neurons?

The clot-busting drug tissue plasminogen activator (tPA) is currently the only FDA-approved therapy for acute stroke. However, increasing evidence suggests that tPA can also contribute to excitotoxic neuronal damage in animal models of stroke. (pages 59–64)

STEPHEN F. TRAYNELIS[1] & STUART A. LIPTON[2]

Stroke, which results from a rupture or obstruction (as by a clot) of an artery of the brain, causes devastating complications and represents a global health problem. According to the National Center for Health Statistics, over 3.3 million people in the United States alone suffer from stroke symptoms. The clot-busting blood protease tissue plasminogen activator (tPA), which has been successfully used to treat myocardial infarction, has been approved for treatment of occlusive stroke. However, tissue-plasminogen activator (tPA) is also believed to have direct effects on neurons, an important factor to consider in evaluating tPA's therapeutic potential for stroke victims. In this issue, Nicole *et al.*[1] report that the proteolytic activity of tPA affects neuronal N-methyl-D-aspartate (NMDA) receptor-mediated signaling.

In the blood, tPA converts plasminogen to plasmin, a protease that dissolves blood clots[2]. As the first treatment targeted at the precipitating event in occlusive stroke (blood vessel blockage), tPA represents a significant therapeutic step forward. However, results from murine stroke models suggest that tPA may also mediate neuronal death[3–6]. In a mouse model of stroke using an intraluminal suture, rather than a blood clot, to cause temporary occlusion of the middle cerebral artery, intravenous injection of tPA produced larger infarcts, indicating that tPA can increase stroke-induced injury[6]. Moreover, in the same model, tPA-deficient mice exhibited approximately 50% smaller cerebral infarcts than wild-type mice with similar genetic backgrounds. Although there has been some controversy surrounding these results, the work has been replicated and several different groups have reported that in addition to its beneficial clot-busting action, tPA can induce neuronal damage[4,5,7,8].

A large amount of basic research has recently been focused on the role of tPA in neuronal function and cell survival. The findings of Nicole *et al.*[1] expand the scope of tPA's complex effects by showing that it can potentiate NMDA-receptor–mediated neurotoxicity. The NMDA receptor is a glutamate-gated ion channel that is highly permeable to Ca^{++}. However, excessive influx of Ca^{++} via this route can lead to a series of dire cellular consequences, including free radical formation, injury and cell death.

Nicole *et al.*[1] used co-immunoprecipitation experiments to demonstrate an apparently direct association between tPA and the NR1 subunit of the NMDA receptor. They conclude that tPA contributes to overactivation of NMDA receptors and exacerbation of neuronal death. Activation of the thrombin receptor PAR1 has also been shown to potentiate NMDA receptor function[9], so a link between blood proteases and NMDA receptors has already been established.

Nicole *et al.*[1] also observed that tPA cleaves the NR1 subunit of the NMDA receptor, removing a fragment of approximately 15–20 kD from its amino terminus (Fig. 1). Ca^{++} imaging results suggest that this cleavage leads to the increased activity of the NMDA receptor. The authors, however, have not ruled out the possibility that the co-immunoprecipitation of NR1 and tPA is accompanied by other unidentified proteins that could be involved in the cleavage of the NR1 subunit. An analysis of recombinant NR1 proteolytic cleavage fragments, along with an NR1 mutational analysis, could be used to identify tPA's cleavage site. Interestingly, the serine protease thrombin also cleaves the NR1 subunit in both neurons and recombinant receptors[9], suggesting NR1 may have structural determinants that enhance its interactions with blood-derived proteases.

It is tempting to extrapolate these results into a simple pathway in which tPA potentiation of NMDA-receptor function alone accounts for the induction of neuronal death; however, there are many other effects of tPA that merit consideration. For example, tPA has been observed to interact with the low-density lipoprotein receptor-related protein to influence neuronal function[10]. In addition, tPA and any plasmin that is formed by tPA proteolysis of plasminogen may cleave or activate other cell surface substrates. These include laminin, matrix metalloproteinases[5] and elements of the immune complement system, which would affect cell adhesion, function and survival after NMDA receptor overactivation.

Furthermore, Nicole *et al.*[1] have not provided evidence that tPA cleavage of NR1 directly alters NMDA receptor func-

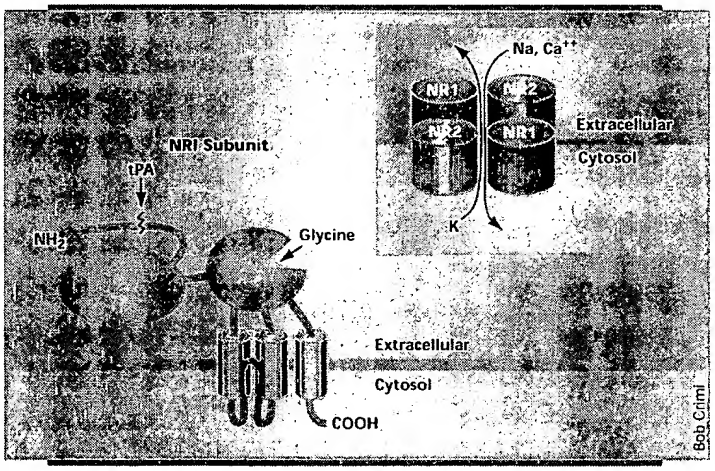

Fig. 1 The NMDA receptor is probably composed of four subunits (inset), one of which, NR1, is illustrated here. NR1 also contains a glycine binding site and an intracellular C-terminus. Nicole *et al.*[1] report that tPA cleaves the NR1 subunit near the N-terminus. This truncated NMDA receptor potentiates the Ca^{++} influx by an unidentified mechanism. The authors suggest that tPA cleavage and increased Ca^{++} influx enhances NMDA receptor-mediated neuronal damage.

tion. NR1 cleavage may inactivate the receptor, have no effect on receptor function, or somehow enhance receptor activity. Future studies must directly assay the effects of tPA through patch-clamp recordings of different cells that express wild-type NMDA receptors, a truncated recombinant form of the NMDA receptor, or a mutated recombinant NMDA receptor that is resistant to tPA proteolysis.

It will also be important to elucidate the mechanism by which alteration of NMDA receptor function affects neuron survival. Nicole *et al.*[1] report that tPA treatment enhances the NMDA-evoked Ca^{++} influx, which could be blocked by co-application of an NMDA receptor antagonist. If accumulation of intracellular Ca^{++} underlies the enhanced neuron death observed in the *in vitro* excitotoxicity assay, these findings suggest that neuronal survival hangs in a very precarious balance, such that even modest increases in the Ca^{++} load induce cell death. Given these questions, the picture of just what tPA is doing seems likely to get murkier before it becomes clear.

Nevertheless, the study suggests interesting new molecular relationships and generates working hypotheses to frame important questions. Despite the need for additional work to understand the link between tPA, NMDA receptors and cell death, the findings are an important step forward. The observed interactions between NR1 and tPA are intriguing from both a biochemical and functional perspective, since tPA has been shown to be released by neuronal PC12 cells in a Ca^{++} dependent fashion[11]. If we are able to eventually understand the mechanism by which tPA interacts with neurons to induce neurodegeneration, we may identify new targets for drugs that promote neuroprotection during stroke therapy.

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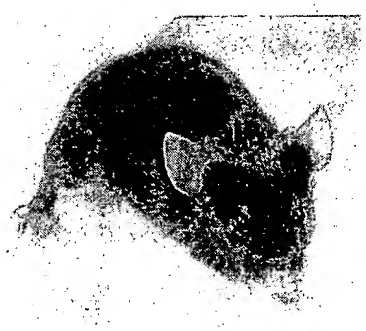
Amyloid β vaccination: reduced plaques and improved cognition

Studies in three different transgenic mouse models suggest that the amyloid β-protein contributes to memory loss in Alzheimer disease. Immunization with an amyloid β-peptide fragment reduces learning and memory impairments in mice, and this approach may eventually be used to prevent and/or treat this disease in people.

STEVEN G. YOUNKIN

Alzheimer disease (AD) is the most common form of dementia in the elderly. Currently there is no effective treatment for this disorder, which is projected to affect 22 million people worldwide by 2025. Two lesions (senile plaques and neurofibrillary tangles) are invariably found in the brains of AD patients. Neurofibrillary tangles are composed primarily of abnormally phosphorylated τ-proteins. The major protein component of senile plaques is the amyloid β-protein (Aβ), a secreted protein released through stepwise proteolytic cleavage of the amyloid β-protein precursor (APP). Most Aβ has 40 amino acids ($Aβ_{40}$) but a minor, slightly longer form ($Aβ_{42}$) is believed to be the major pathogenic species in AD. Three studies, published in the 21/28 December issue of *Nature* by Chen *et al.*[1], Janus *et al.*[2] and Morgan *et al.*[3], provide new evidence that learning and memory defects are related to Aβ accumulation, and two of the reports[2,3] show that Aβ immunization can reduce these deficits.



Memory deficits in Tg2576 transgenic mice, which overexpress mutant APP, can be reversed by $Aβ_{42}$ vaccination.

The abundant evidence that Aβ aggregation is an essential early event in AD pathogenesis has prompted an intensive search for therapeutics that target Aβ. This search has been aided immeasurably by transgenic mouse models of AD. These models overexpress mutant forms of APP and Presenilin 1 (PS1), which cause early onset familial AD. This overexpression causes an age-dependent accumulation of Aβ plaques that are quantitatively and qualitatively similar to those of the AD brain, but does not reproduce the complete phenotype observed in human AD brain, as neurofibrillary tangles do not develop and there is considerably less neuron and synapse loss. The current AD transgenic models are an excellent tool for evaluating therapies that target Aβ, and they can be used to determine if age-dependent

Adding to the Effectiveness of Intravenous Tissue Plasminogen Activator for Treating Acute Stroke

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Intravenous tissue plasminogen activator (rt-PA) remains the only approved therapy for acute ischemic stroke with demonstrated effectiveness in phase 3 clinical trials.[1] Unfortunately, the utilization of this treatment is limited by a 6% risk of symptomatic brain hemorrhage[2] and a brief 3-hour time window of efficacy from symptom onset to treatment.[3] Furthermore, its effectiveness is limited for several reasons; intravenously administered rt-PA often fails to lyse large clots,[4,5] arteries reocclude in about a third of cases,[5] flow may remain stagnant in the microcirculation despite clot lysis,[6,7] and cellular injury may continue despite reperfusion.[8]

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Attempts to augment the effect of IV rt-PA have so far been only partially successful. The most promising results have occurred with endovascular techniques that deliver smaller doses of lytic drugs, energy-producing catheters, or mechanical devices directly into the clot to achieve more complete lysis. Pro-urokinase delivered directly into proximal occlusions of the middle cerebral artery on average 5.3 hours after stroke onset was able to achieve lysis in 66% of cases with improved outcome (over heparin alone) in a phase 2 trial.[9] Similarly, if IV rt-PA is followed by intra-arterially administered rt-PA (average 3.6 hours after stroke onset), complete or partial recanalization occurred in 56% with improved outcome compared with historical placebo-treated controls.[10] Both of these studies need replication with a larger sample and head-to-head comparison against IV rt-PA alone. Studies with various energy-producing and mechanical disruption catheters are just starting. All of these endovascular techniques have the limitations of expense, available expert manpower, and most importantly, time.

Antithrombotic therapies alone have not proven effective in acute stroke patients, and they do increase bleeding risk. To date, they have not been tested in conjunction with lytics. Heparin, low molecular weight heparins, and heparinoids have proven ineffective in acute stroke patients in recent trials,[11–13] a study of the direct thrombin inhibitor Argatroban (GlaxoSmithKline) has just been stopped because of futility,[14] and aspirin alone is of limited benefit.[11,15] A phase 2 study of the glycoprotein IIb/IIIa receptor antagonist abciximab was reported at an international meeting in February 2003, and phase 3 studies are planned based on this preliminary safety study.[16]

Cytoprotective therapies targeting perturbed ion channels, activated neurotransmitter receptors, and inflammation after acute stroke have also been neutral or negative, but these drugs have only rarely been linked with lytics in a combined strategy.[17]

It is logical to consider combinations of therapeutic strategies for treating this difficult condition. It makes sense to link lytic therapy with antithrombotic drugs to augment and speed lysis, reduce reocclusion, and improve microcirculatory flow in the brain after ischemic stroke. If this could be achieved, we might expect better clinical outcomes in patients with large clots treated early, and, if faster lysis is achieved, perhaps benefit for those patients in whom treatment cannot occur until the end of the treatment window.

The study reported in this issue of *Circulation* by Zhang and colleagues[18] demonstrating the benefits of combining IV rt-PA with a glycoprotein IIb/IIIa receptor antagonist supports this concept. Highlights of this study include a unique embolic stroke model in rats that is amenable to treatment with lytic drugs, a positive effect on both histological and behavioral outcomes when the drug combination was delayed until 4 hours after stroke (when neither treatment alone had an effect), no increase in bleeding compared with rt-PA alone, and confirmation of benefit on microcirculatory flow.

Combined with an earlier study from the same laboratory showing similar results with Argatroban,[19] data have shown that it is time to move ahead with pilot human studies to see if these combined approaches will result in clinically meaningful benefit compared with IV rt-PA alone. This may be easier to detect in patients treated 2 to 4 hours after stroke onset when the benefit of IV rt-PA alone is less apparent and when most stroke patients who are eligible for early treatment arrive in the emergency department. Increased bleeding was not seen with the combination in these experimental studies, and in fact, when the rt-PA dose was reduced by 50% in the combination, there were no hemorrhages and clinical benefit was preserved. Despite these reassuring results, it will be essential to closely monitor bleeding complications in future stroke trials of any combination strategy.

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KEY WORDS: Editorials ■ stroke ■ plasminogen activators

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Activated Protein C Prevents Neuronal Apoptosis via Protease Activated Receptors 1 and 3

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Summary

Activated protein C (APC), a serine protease with anticoagulant and anti-inflammatory activities, exerts direct cytoprotective effects on endothelium via endothelial protein C receptor-dependent activation of protease activated receptor 1 (PAR1). Here, we report that APC protects mouse cortical neurons from two divergent inducers of apoptosis, N-methyl-D-aspartate (NMDA) and staurosporine. APC blocked several steps in NMDA-induced apoptosis downstream to nitric oxide, i.e., caspase-3 activation, nuclear translocation of apoptosis-inducing factor (AIF), and induction of p53, and prevented staurosporine-induced apoptosis by blocking caspase-8 activation upstream of caspase-3 activation and AIF nuclear translocation. Intracerebral APC infusion dose dependently reduced NMDA excitotoxicity in mice. By using different anti-PARs antibodies and mice with single PAR1, PAR3, or PAR4 deletion, we demonstrated that direct neuronal protective effects of APC in vitro and in vivo require PAR1 and PAR3. Thus, PAR1 and PAR3 mediate anti-apoptotic signaling by APC in neurons, which may suggest novel treatments for neurodegenerative disorders.

Introduction

Activated protein C (APC) is a serine protease with systemic anticoagulant and anti-inflammatory activities (Griffin et al., 2002). The best-known activity of APC is its anticoagulant activity. However, the direct effects of APC on cells are subject to increasing attention. Recent studies demonstrate that APC reduces organ damage in animal models of sepsis (reviewed in Griffin et al., 2002) and in humans with severe sepsis (Bernard et al., 2001), represses apoptosis in the developing placenta (Isermann et al., 2003), and protects brain from ischemic injury (Shibata et al., 2001; Cheng et al., 2003). It also prevents p53-dependent apoptosis in hypoxic endothelium in vitro and in vivo through endothelial protein C receptor (EPCR)-dependent activation of protease activated receptor 1 (PAR1) (Cheng et al., 2003). Its direct effects on endothelial cells require EPCR and PAR1 (Riewald et al., 2002; Cheng et al., 2003; Domotor et al., 2003; Mosnier and Griffin, 2003).

It is not clear, however, whether APC can protect directly perturbed neurons by activating PARs, or whether APC's neuroprotection is secondary to its antithrombotic and anti-inflammatory activities or its anti-apoptotic activity in brain endothelium (Cheng et al., 2003). PARs, G protein-coupled receptors, are activated by proteolytic cleavage by various proteases that results in an intramolecular tethered ligand that triggers intracellular signaling (Coughlin, 2000; Coughlin and Camerer, 2003; Bahou, 2003). PAR1 was first discovered as a human platelet thrombin receptor (Vu et al., 1991), and today four homologous PARs are known in man and mice (Coughlin and Camerer, 2003; Bahou, 2003). The various PARs are differentially expressed in various cells and tissues, and all four PARs are expressed in the central nervous system (Striggow et al., 2001). The effects of activation of each PAR by specific protease in any given organ or cell type generally remain to be elucidated.

We hypothesized that APC's interaction with PARs in neurons is neuroprotective. To test this hypothesis, we studied two divergent inducers of neuronal apoptosis: N-methyl-D-aspartate (NMDA), using in vitro (Bonfoco et al., 1995) and in vivo (Ayata et al., 1997) models of NMDA-induced neuronal injury, and staurosporine (Budd et al., 2000). Overstimulation of NMDA receptors is implicated in stroke, brain trauma, and neurodegenerative disorders such as Alzheimer's disease and Huntington's disease (Le and Lipton, 2001; Kemp and McKernan, 2002). The mechanisms involved in NMDA-induced neuronal apoptosis include generation of nitric oxide (Dawson et al., 1991; Ayata et al., 1997), activation of caspase-3 and -7 (Du et al., 1997; Tenneti and Lipton, 2000; Okamoto et al., 2002), nuclear translocation of apoptosis-inducing factor (AIF) and activation of PARP-1 (poly(ADP)-ribose polymerase-1) (Yu et al., 2002), and increases in p53 and *Bax* (Sakhi et al., 1997; Uberti et al., 1998; Djebaili et al., 2000; Jordan et al., 2003). Staurosporine, but not NMDA, targets caspase-8 upstream of caspase-3 activation (Budd et al., 2000). It may induce apoptosis independent of p53 (Jordan et al., 2003; Villunger et al., 2003), although involvement of p53 at later stages of staurosporine-mediated apoptosis has been suggested (Tieu et al., 1999; Alves da Costa et al., 2003). Here, we show that APC protects against NMDA-induced apoptosis in mouse cortical neurons by blocking caspase-3 activation, nuclear translocation of AIF, and induction of p53, and prevents staurosporine-induced apoptosis by blocking caspase-8 activation upstream of caspase-3 activation and AIF nuclear translocation. Intracerebral infusion of APC dose dependently reduced NMDA excitotoxicity in mice. By using different anti-PARs antibodies and mice with single PAR1 (Coughlin, 2000), PAR3,

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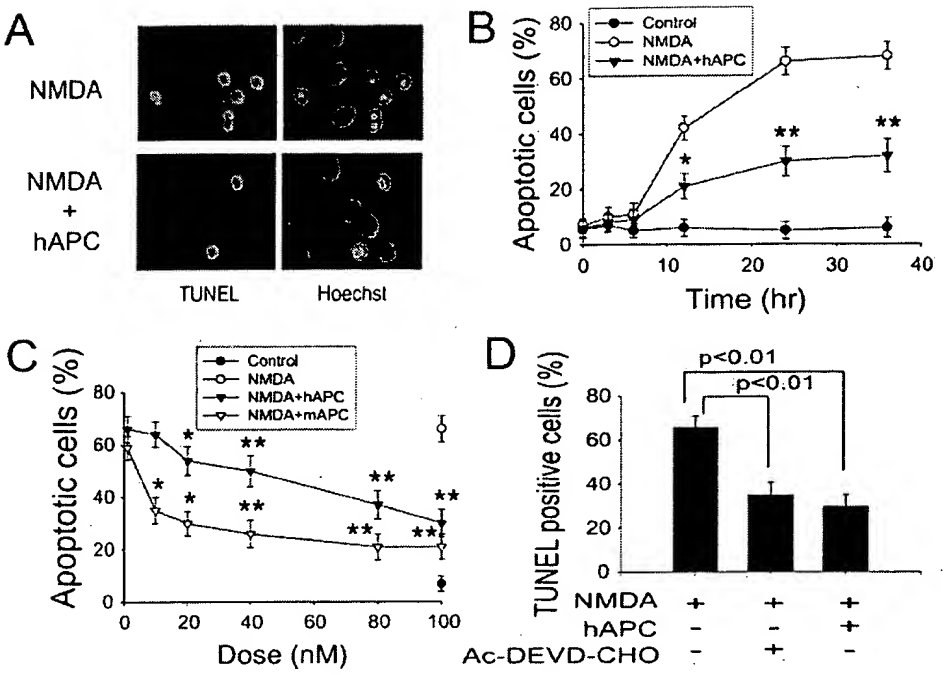

Figure 1. APC Blocks NMDA-Induced Apoptosis in Mouse Cortical Neurons

(A) Immunostaining for TUNEL and Hoechst 24 hr after NMDA in the absence or presence of human APC (hAPC, 100 nM).

(B) Effect of hAPC (100 nM) on NMDA-induced apoptosis as a function of time; apoptotic cells were quantified with TUNEL and Hoechst. Control spontaneous rate of apoptosis is also illustrated (solid points, control).

(C) Dose-dependent neuroprotective effects of hAPC and recombinant mouse APC (mAPC) at 24 hr of NMDA; control rate of apoptosis (solid point) and rate of apoptosis in the presence of NMDA only (open point) are also illustrated.

(D) TUNEL-positive cells in experiments as in (A) in the presence of Ac-DEVD-CHO (50 μM) or hAPC (100 nM). *$p < 0.05$, **$p < 0.01$ compared with values in the absence of APC.

or PAR4 deletion (Nakanishi-Matsui et al., 2000), we demonstrated that direct neuronal protective effects of APC in vitro and in vivo require both PAR1 and PAR3.

Results

Figure 1A illustrates that the number of TUNEL-positive cells with apoptotic nuclear changes in NMDA-perturbed mouse cortical neurons was significantly reduced by human plasma-derived APC (100 nM) 24 hr after NMDA challenge. APC reduced the number of TUNEL-positive cells in a time-dependent manner including earlier and later time points at 12 and 36 hr of exposure to NMDA (Figure 1B). Figure 1C shows dose-dependent neuronal protection by human and mouse recombinant APC. The IC_{50} values for reducing neuronal apoptosis for human and mouse APC were 49 and 5 nM, respectively, confirming significantly higher efficacy of the species homologous mouse APC, consistent with a recent report in a mouse stroke model (Cheng et al., 2003). Ac-DEVD-CHO, a specific inhibitor of caspase-3 activation, blocked NMDA-induced apoptosis (Figure 1D), consistent with several reports (Du et al., 1997; Tenneti and Lipton, 2000; Budd et al., 2000; Okamoto et al., 2002). Inhibition by Ac-DEVD-CHO was comparable to that obtained by APC raising a possibility that APC may block pro-apoptotic caspase-3 activation.

In response to NMDA, the number of caspase-3-positive cells that were also TUNEL positive (Figure 2A) were significantly reduced by >60% by APC (Figure 2B). APC significantly reduced capsase-3 activity in a time-dependent manner (Figure 2C) but did not affect caspase-8 activity (data not shown), consistent with the previous report (Budd et al., 2000). Next, we confirmed nuclear translocation of AIF in response to NMDA (Yu et al., 2002) at 18 and 24 hr and demonstrated that both APC and/or Ac-DEVD-CHO efficiently block AIF translocation (Figures 2D and 2E).

We confirmed that NMDA induces generation of cGMP, which is an indirect sensitive measure of NO production (Figure 3A; Dawson et al., 1991). Generation of cGMP was inhibited by nitric oxide synthase (NOS) inhibitors N-arginine and L-NAME as reported (Dawson et al., 1991), but not by APC (Figure 3A). L-arginine reversed the effect of NOS inhibitors (not shown). In response to NMDA treatment, there was an early increase in p53 protein and mRNA (Figures 3B and 3C), possibly related to increased NO production (Morrison et al., 2003; Brüne and Schneiderhan, 2003). The present data demonstrate that NOS inhibitors prevent induction of p53 in NMDA-treated neurons, while L-arginine reverses the effect on NOS inhibitors (Figure 3D), consistent with previous report (Dawson et al., 1991). APC significantly reduced by half the increased levels of p53 protein in nuclear extracts of NMDA-treated neurons (Figures 3B and 3F) and the levels of p53 mRNA transcripts (Figure

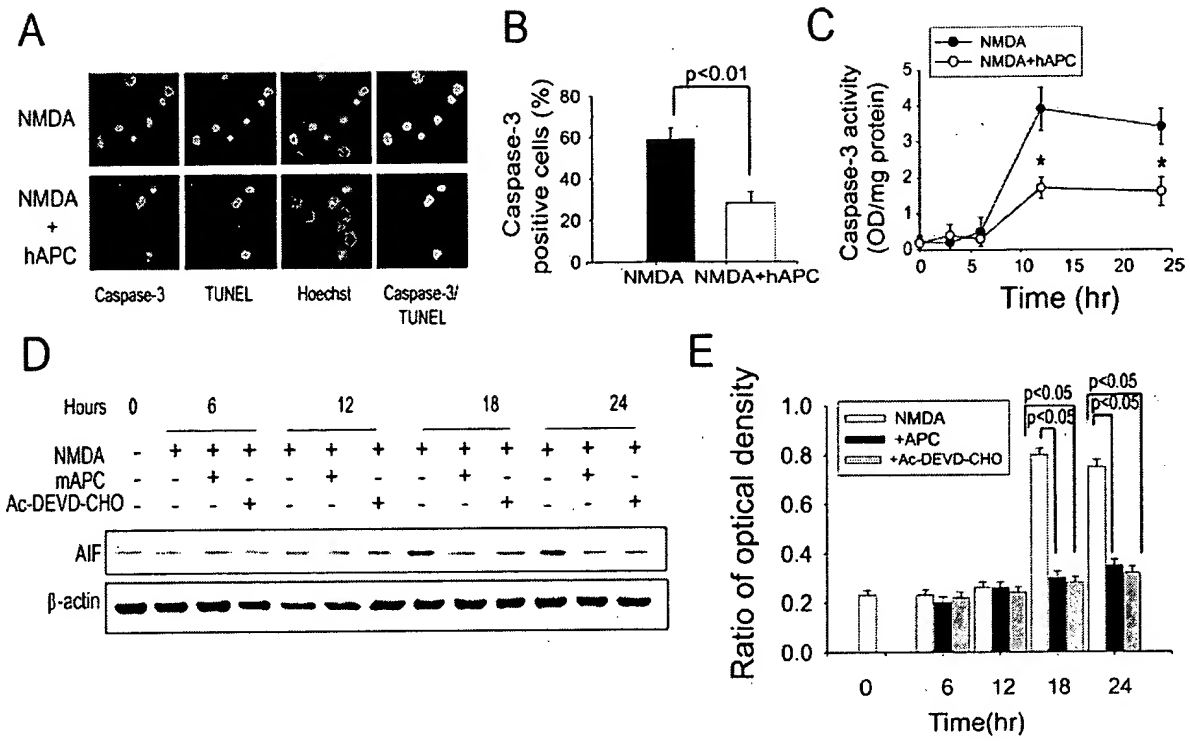

Figure 2. Effects of APC on Caspase-3 and AIF in NMDA-Treated Neurons

(A) Caspase-3, TUNEL, and Hoechst triple staining 24 hr after NMDA in the absence or presence of human APC (hAPC, 100 nM).

(B) Caspase-3-positive cells 24 hr after NMDA with and without hAPC (100 nM).

(C) Caspase-3 activity in cultured cortical neurons; time course after exposure to NMDA ± hAPC (100 nM).

(D) Western blot analysis of AIF in nuclear extracts from NMDA-treated cells and the effects of murine APC (mAPC, 10 nM) and Ac-DEVD-CHO (50 μM) on AIF nuclear translocation.

(E) Intensity of AIF nuclear signal measured by scanning densitometry and effect of mAPC and Ac-DEVD-CHO in experiments as in (D). Data are mean ± SEM (3–5 independent experiments in triplicate). *$p < 0.05$ compared with values in the absence of APC.

3C). Phosphorylation of p53 on Ser20 or Ser15, which would stabilize the protein by preventing its proteosomal degradation by precluding its binding to the oncoprotein murine double minute-2 (Mdm2), was undetectable either in nuclear extracts or whole cell homogenates (not shown). Changes in Mdm2 protein in response to NMDA were undetectable, and direct effects of APC on Mdm2 protein were not observed (not shown).

Figures 3E and 3F show that NMDA increases *Bax* as suggested (Djebaili et al., 2000) and decreases *Bcl-2*. Consistent with p53 blockage, APC blunted the increases in *Bax* and the decreases of *Bcl-2* (Figures 3E and 3F), thus normalizing the *Bax/Bcl-2* ratio. Since NMDA may increase the levels of nuclear factor kB (NF-κB) (McInnis et al., 2002) that can be either anti-apoptotic or pro-apoptotic (Ryan et al., 2000), we tested whether the observed changes in p53 expression are downstream to NF-κB. Under present experimental conditions, NMDA did not induce NF-κB translocation into the nucleus in cortical cells (Figure 3G), whereas the positive control *E. coli* lipopolysacharide did cause NF-κB translocation in umbilical vein endothelium (Figure 3G).

We also studied whether APC affects NMDA receptor structure and function. Tissue plasminogen activator (tPA) action on NMDA receptors may involve proteolysis of the NR1 subunit either due to tPA direct action (Nicole et al., 2001) or indirectly due to generation of plasmin (Matys and Strickland, 2003). In contrast to reported effects for tPA (Nicole et al., 2001) and/or plasminogen (Matys and Strickland, 2003), APC did not cleave the NMDA receptors subunits (Figure 3H) and also did not alter NMDA-stimulated fura-2 fluorescence signal (not shown). Thus, APC does not modify the structure nor does it block the NMDA receptors. Since blocking NMDA receptors or modifying their structure could be deleterious, the current strategies are centered on blocking NMDA-induced apoptosis downstream to the NMDA receptors (Aarts et al., 2002).

To define the specificity of APC's neuroprotection, we demonstrated that neither mutant Ser360Ala-APC nor protein C zymogen could protect neurons from NMDA-induced apoptosis, and that heat denaturation or anti-APC IgG abrogated APC's activity (Figure 4A). This confirms that the active site serine of APC is necessary for neuronal protection and suggests that PARs may be involved. To test the role of PARs, first we studied the neuronal protective effects of mouse recombinant APC in the presence of various anti-PAR antibodies (Figure 4B). The presence of PAR1, PAR2, PAR3, and PAR4 in the present model has been confirmed by positive immunostaining on mouse cortical neurons (not shown), as reported (Striggow et al., 2001). Antibody blockage

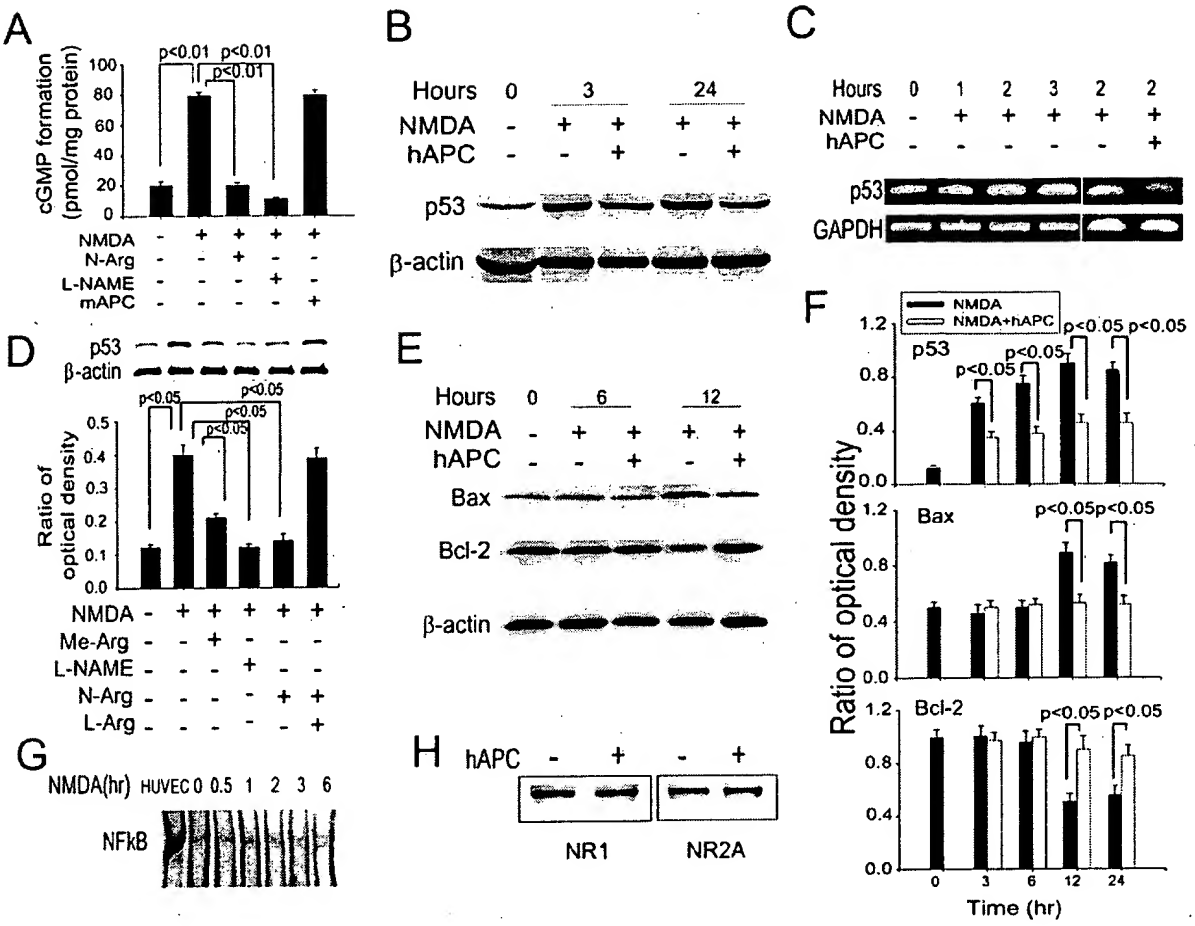

Figure 3. APC Blocks p53 Induction in NMDA-Treated Neurons

(A) cGMP formation 10 min after NMDA application in the presence of mouse APC (mAPC, 10 nM) and N-arginine (100 μM) and L-NAME (500 μM).

(B and C) Western blots (B) for p53 in nuclear protein extracts and RT-PCR (C) for p53 mRNA from NMDA-treated cells in the absence or presence of human APC (hAPC, 100 nM).

(D) Western blots for p53 in the presence of Me-arginine (100 μM), L-NAME (500 μM), and N-arginine (100 μM) and reversal by L-arginine (100 μM).

(E) Western blots for *Bax* and *Bcl-2* on whole-cell extracts from experiments as in (B).

(F) Intensity of p53, *Bax*, and *Bcl-2* signals normalized to β-actin in response to NMDA and the effect of hAPC (100 nM).

(G) NF-κB DNA binding activities following exposure of neurons to NMDA; HUVEC exposed to LPS (*E. coli*) was a positive control.

(H) Western blots of NMDA receptor subunits NR1 and NR2A in membrane fractions 24 hr after treatment with hAPC (100 nM) for 24 hr. Data are mean ± SEM (3–5 independent experiments for each studied time point).

of the PAR1 cleavage site, but not of the PAR2 cleavage site, caused significant (~70%) loss of APC-mediated neuronal protection (Figure 4B). In negative controls, antibodies against the N-terminal, cleavage-independent regions of PAR1 and PAR2, or against the C terminus of PAR4, were without effect (Figure 4B). An antibody against the extracellular N-terminal 103 amino acids of PAR3 significantly blocked APC-mediated neuronal protection (~65%), in contrast to a control anti-PAR3 antibody against the C terminus of PAR3 (Figure 4B). Combination of antibodies that each partially blocked activation of PAR1 and PAR3 completely abrogated APC's neuronal protective effects (Figure 4B). We also demonstrated that PAR1 agonist peptide, TFLLRNPNDK, but not the PAR2 agonist peptide (SLIGRL), protected neurons against NMDA-induced apoptosis (Figure 4B). The essential role of PAR1 was confirmed by a complete loss of APC-mediated neuronal protection in neurons cultured from PAR1 null mice (Figure 4C).

To determine whether APC's in vivo neuroprotection requires PAR1 and PAR3, we studied the effects of intracerebral APC on NMDA-induced lesions in mice after stereotactic injections of NMDA into the caudate nucleus (Ayata et al., 1997). Local administration of mouse APC (0.2 μg) significantly reduced by >70% the volume of NMDA-induced lesions determined at 48 hr after NMDA injection (Figures 5A and 5B); this neuronal protective effect of APC was dose dependent (Figure 5B), similar to the in vitro results (Figure 1C). There was >70% loss of APC neuroprotective effect in vivo in mice treated with APC plus anti-PAR1 antibody (Figure 5C) and >65% loss of APC-mediated neuroprotection in the presence of anti-PAR3 antibody. As in vitro (Figure 4B), combination of antibodies that each partially blocked activation of PAR1 and PAR3 abolished APC's neuroprotective effects in vivo (Figure 5C).

Studies in PAR1 null, PAR3 null, and PAR4 null mice of NMDA-induced lesions in the absence and presence

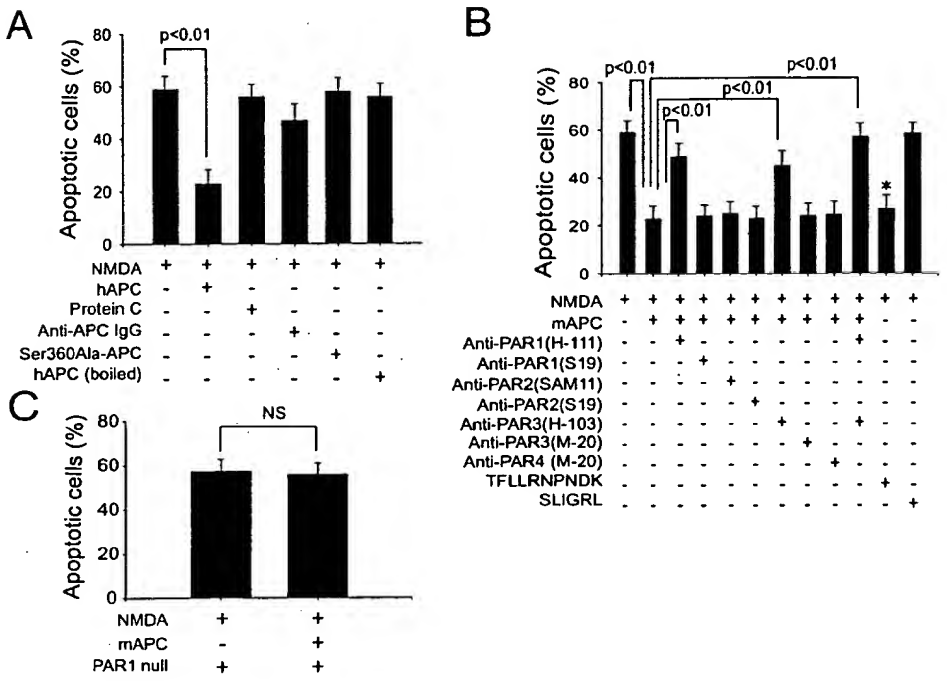

Figure 4. PAR1 and PAR3 Mediate APC's Neuroprotection in NMDA-Treated Neurons

(A) Cortical neurons treated with NMDA in the presence of vehicle, human APC (hAPC), protein C zymogen, anti-APC IgG (C3), Ser360Ala-APC, and boiled hAPC at 100 nM; apoptotic cells were quantified as in Figure 1.

(B) Cortical neurons treated with NMDA and incubated with mouse APC (mAPC, 10 nM) and various cleavage site blocking PAR1, PAR2, and PAR3 antibodies (20 μg/ml); N-terminal blocking PAR1 and PAR2 antibodies and C-terminal blocking PAR3 and PAR4 antibodies (20 μg/ml) were negative controls. TFLLRNPNDK (10 μM) and SLIGRL (100 μM), PAR1 and PAR2 agonist peptides, respectively.

(C) Lack of mAPC (10 nM) effect on NMDA-induced apoptosis in neurons cultured from PAR1 null mice. Values are mean ± SEM (n = 3–5 independent experiments in triplicate). *p < 0.01, NMDA versus NMDA + TFLLRNPNDK; NS, nonsignificant.

of APC indicated that APC-mediated neuroprotection was lost by approximately 84% and 72% in PAR1 null and PAR3 null mice relative to controls, respectively, while deletion of PAR4 gene did not result in loss of APC-mediated neuroprotection (Figure 5D).

To test whether APC can also protect neurons against an alternative apoptotic stimulus distinct from NMDA, we studied its neuroprotective effects in a model of staurosporine-induced apoptosis associated with activation of caspase-8, which is not involved in NMDA-mediated apoptosis (Budd et al., 2000). Our data indicate that APC can prevent staurosporine-mediated apoptosis (Figure 6A) by blocking activation of caspase-8 (Figure 6B) upstream of caspase-3 (Figure 6C). We confirmed that selective inhibitors of caspase-8 (z-IEDT-fmk) and caspase-3 (Ac-DEVD-CHO) block staurosporine-induced apoptosis (Figure 6A) and that inhibition of caspase-8 activation results in blockade of caspase-3 activation (Figure 6B), as reported (Budd et al., 2000). APC prevented AIF translocation from mitochondria to nucleus downstream to caspase-8 and -3 (Figures 6D and 6E), as demonstrated by inhibition of the AIF mitochondrial to nuclear translocation by both z-IETD-fmk and Ac-DEVD-CHO (Figures 6D and 6E). In addition, we confirmed that activation of caspase-3 by staurosporine does not require p53, as demonstrated by unchanged levels of p53 protein (Figure 6F) prior and at the time of increased caspase-3 activation (Figure 6C). This result is consistent with a recent report demonstrating staurosporine-induced activation of caspase-3 in p53-deficient cells (Paitel et al., 2004).

Next, we studied whether PARs are involved in APC's neuroprotection in staurosporine model. As in NMDA model, both mouse and human APC protected mouse neurons against staurosporine insult, but higher dose of human APC was needed for the same level of protection. We demonstrated that neither mutant Ser360Ala-APC nor protein C zymogen could protect neurons from staurosporine-mediated apoptosis, and that heat denaturation or anti-APC IgG abrogated APC's activity (Figure 7A). Antibody blockage of the PAR1 cleavage site, but not PAR2 cleavage site, caused 75% loss of APC-mediated cytoprotection, while an antibody against the extracellular N-terminal 103 amino acids of PAR3 blocked by 69% APC-mediated neuroprotection (Figure 7B). Combination of antibodies that each partially blocked activation of PAR1 and PAR3 almost completely inhibited APC's neuroprotective effects (Figure 7B), confirming that PAR1 and PAR3 are required for APC's neuroprotection against staurosporine similar as they are required for APC's neuroprotection against NMDA toxicity in vitro (Figures 4B and 4C) and in vivo (Figures 5C and 5D).

Discussion

Recent studies in endothelial cells show that APC activates PAR1 in an EPCR-dependent manner, alters intracellular signaling, causes alterations in gene expression profiles, and exerts direct anti-apoptotic effects (Joyce et al., 2001; Riewald et al., 2002; Cheng et al., 2003;

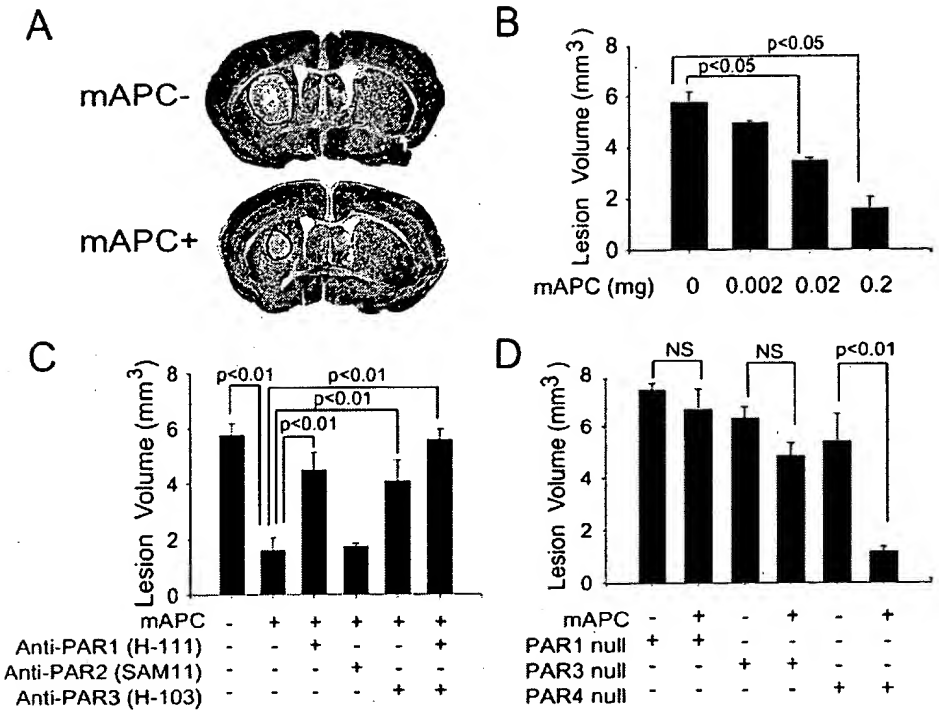

Figure 5. PAR1 and PAR3 Mediate APC's Neuronal Protection against NMDA In Vivo

(A) Coronal sections of mouse brains infused with NMDA in the absence of mouse APC (mAPC−) or presence of mAPC (mAPC+; 0.2 μg).

(B) Dose-dependent protective effect of mAPC on NMDA-induced injury in mouse striatum.

(C) Effects of different anti-PAR antibodies on NMDA lesion volumes in the absence or presence of mAPC (0.2 μg); antibodies were used at 0.2 μg.

(D) NMDA lesion in PAR1 null, PAR3 null, and PAR4 null mice in the absence and presence of mAPC (0.2 μg). Values are mean ± SEM, n = 3–5 mice; NS, nonsignificant.

Domotor et al., 2003; Mosnier and Griffin, 2003; Ruf, 2003). Here, we present evidence that APC is able to influence the function of neurons through PAR1 and PAR3 by interfering with several pathways of NMDA-induced and staurosporine-induced apoptotic cascades. Our data suggest that APC does not interfere with NMDA-induced NO generation (Dawson et al., 1991; Ayata et al., 1997), but prevents both caspase-3 activation (Du et al., 1997; Tenneti and Lipton, 2000; Okamoto et al., 2002) and nuclear translocation of AIF, a mitochondrial flavoprotein, released in response to NMDA (Yu et al., 2002). In case of staurosporine-induced apoptosis, APC prevents activation of caspase-8 upstream to activation of caspase-3 and nuclear translocation of AIF.

As AIF induces apoptotic changes in purified nuclei in the presence of caspase inhibitors (Susin et al., 1999), it has been assumed that its function is caspase independent. However, AIF is released from mitochondria after cytochrome c release subsequent to activation of caspases (Arnoult et al., 2002). Consistent with the present findings in NMDA-mediated and staurosporine-mediated neuronal apoptosis, it has been recently shown that AIF acts downstream to caspases in both *C. elegans* (Wang, 2001) and human cells (Arnoult et al., 2002; Penninger and Kroemer, 2003; Gabriel et al., 2003). A recent report failed to demonstrate NMDA-induced caspase-3 activation (Yu et al., 2002) in contrast to the present and several previous reports (Du et al., 1997; Tenneti and Lipton, 2000; Budd et al., 2000; Okamoto et al., 2002). The differences could be related to the different amplitude and duration of NMDA-induced signaling, which likely may influence the prevailing apoptotic cascade.

Several lines of evidence suggest that p53 is a key upstream initiator of neuronal cell death in the mature nervous system following diverse number of insults, e.g., excitotoxic injury including NMDA (Uberti et al., 1998; Djebaili et al., 2000; Jordan et al., 2003), ischemia, seizure activity, and X radiation (Morrison et al., 2003). On the other hand, diverse cytotoxic insults such as cytokine withdrawal, phorbol ester, and staurosporine may induce p53-independent apoptosis in various cell types (Villunger et al., 2003; Jordan et al., 2003). Our data in NMDA-treated neurons suggest that APC blocks transcriptionally dependent p53 induction downstream to NO but upstream of caspase-3 activation. By reducing *Bax/Blc-2* ratio, APC can stabilize the mitochondrial membrane to cytochrome c, thus precluding activation of caspases (Penninger and Kroemer, 2003). On the other hand, AIF release from mitochondria could be p53 dependent and *Bax* dependent (Cregan et al., 2002) or PARP-1 dependent (Yu et al., 2002). Thus, by downregulating p53 and *Bax* in neurons, APC may prevent AIF release from mitochondria. Consistent with this concept, APC-induced increased expression of *Bcl-2* may also prevent AIF release (Susin et al., 1999). Our data in staurosporine model indicate that APC may also prevent

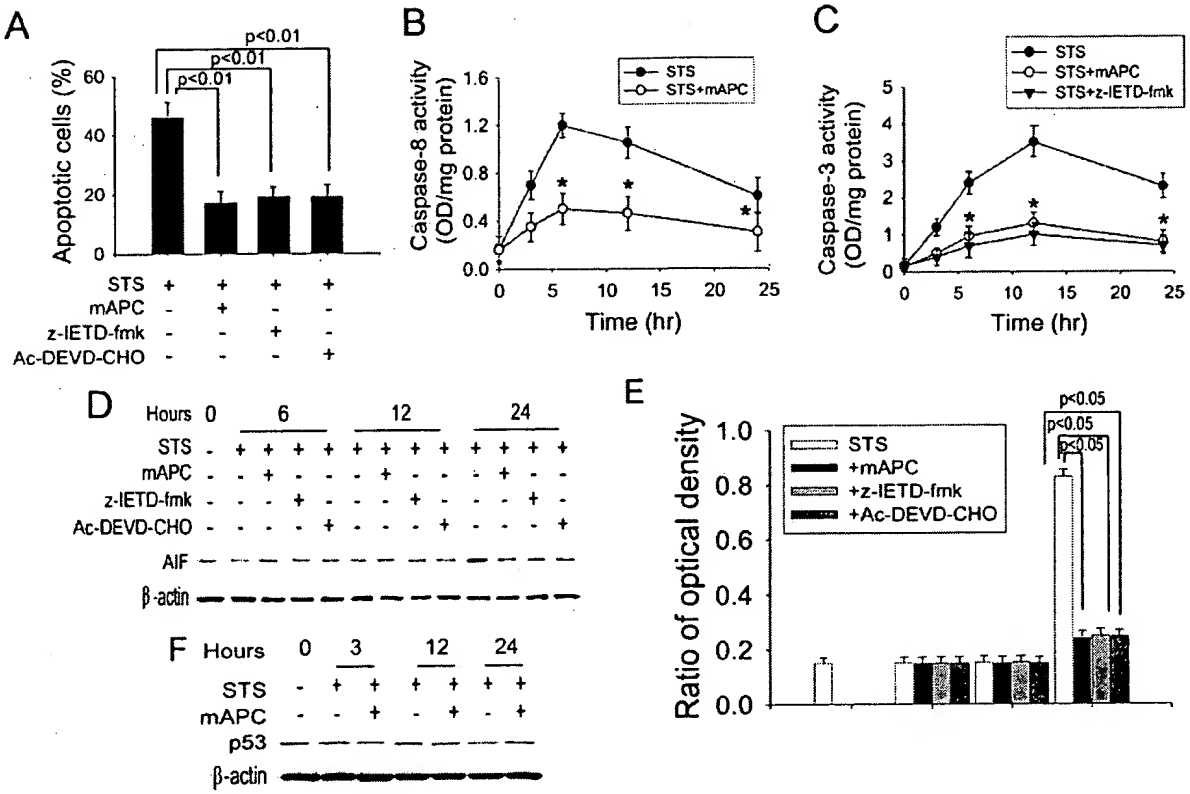

Figure 6. APC Blocks Staurosporine-Induced Apoptosis in Mouse Cortical Neurons

(A) Effect of mAPC (10 nM), z-IEDT-fmk (20 μM), and Ac-DEVD-CHO (50 μM) on staurosporine (STS, 0.5 μM)-induced apoptosis; apoptotic cells were quantified with TUNEL and Hoechst as in Figure 1.

(B) Caspase-8 activity in cultured cortical neurons; time course after exposure to STS ± mAPC (10 nM).

(C) Caspase-3 activity in cultured cortical neurons; time course after exposure to STS in the absence and presence of mAPC (10 nM) and z-IEDT-fmk (20 μM).

(D) Western blot analysis of AIF in nuclear extracts from STS-treated cells and the effects of murine APC (mAPC, 10 nM), z-IEDT-fmk (20 μM), and Ac-DEVD-CHO (50 μM) on AIF nuclear translocation.

(E) Intensity of AIF nuclear signal measured by scanning densitometry and effect of mAPC, z-IEDT-fmk, and Ac-DEVD-CHO in experiments as in (D).

(F) Western blots for p53 in nuclear protein extracts after STS ± mAPC (10 nM). Data are mean ± SEM (3–5 independent experiments in triplicate). *$p < 0.05$ compared with values in the absence of APC or z-IEDT-fmk.

AIF release by activating an alternative anti-apoptotic pathway through blockade of caspase-8 activation upstream of caspase-3 activation, which does not require APC's inhibition of p53-mediated pro-apoptotic signaling.

Our studies indicate that whereas PAR3 and PAR4, but not PAR1, are required for normal action of thrombin on mouse platelets (Nakanishi-Matsui et al., 2000), PAR1 and PAR3, but not PAR4, are required for optimal in vitro and in vivo neuronal protective action of APC. PAR1

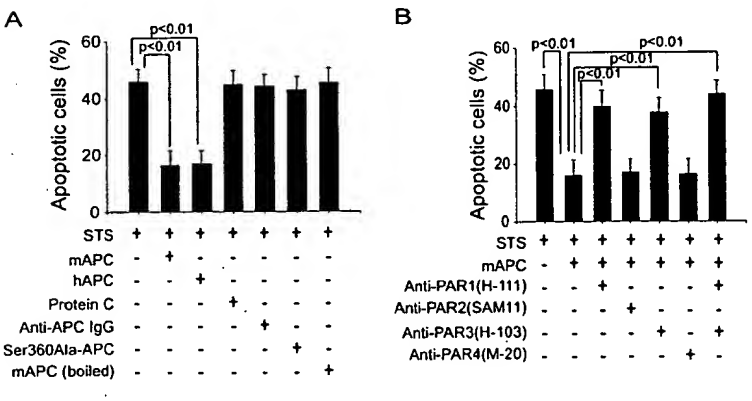

Figure 7. PAR1 and PAR3 Mediate APC's Neuroprotection against Staurosporine

(A) Cortical neurons treated with staurosporine (STS, 0.5 μM) in the presence of vehicle, mouse APC (mAPC, 10 nM), human APC (hAPC, 100 nM)), human protein C zymogen (100 nM), human anti-APC IgG (C3), human Ser360Ala-APC (100 nM), and boiled mAPC (10 nM); apoptotic cells were quantified with TUNEL and Hoechst.

(B) Cortical neurons treated with STS and incubated with mouse APC (mAPC, 10 nM) and cleavage site blocking PAR1, PAR2, and PAR3 antibodies (20 μg/ml); C-terminal blocking PAR4 antibody (20 μg/ml) was negative control. Values are mean ± SEM (n = 3–5 independent experiments in triplicate).

agonist peptide and thrombin at higher concentrations can kill neurons (Donovan and Cunningham, 1998; Striggow et al., 2000), but at concentrations comparable to those used in the present study, the PAR1 agonist protects cortical rat neurons and astrocytes from hypoglycemia and oxygen/glucose deprivation (Donovan and Cunningham, 1998; Striggow et al., 2000). Thus, activation of PAR1 in neurons may be anti-apoptotic, as in the case of APC and very low dose thrombin. The pro-apoptotic activity of higher levels of thrombin might involve the action of thrombin on substrates other than PAR1 (e.g., PAR4) and/or differences in amplitude and duration of PAR1 signaling. Although no data show direct APC cleavage of PAR1 or PAR3 in cells, APC can cleave a synthetic PAR1 N-terminal polypeptide at Arg41, the thrombin cleavage site, at a rate 5000 times slower than thrombin (Kuliopulos et al., 1999). Further studies are needed to clarify if APC directly activates PAR3, if PAR3 is cofactor for PAR1 activation by APC, if PAR3 contributes to PAR heterodimerization, or if PAR3 in neurons acts by a hitherto unknown mechanism.

In summary, this study shows that APC interactions with PAR1 and PAR3 on neurons can protect neurons against divergent inducers of apoptosis, which could be of importance for developing new therapeutic strategies to treat neurodegenerative disorders.

Experimental Procedures

Reagents

N-methyl-D-aspartate (NMDA) was purchased from Sigma (St. Louis, MO) and staurosporine from Calbiochem (San Diego, CA). Human APC, recombinant mouse APC, protein C zymogen, mutant Ser360Ala-APC, and mouse IgG against human APC (C3 antibody) were prepared as reported (Shibata et al., 2001; Cheng et al., 2003; Fernandez et al., 2003). Unless otherwise indicated, for Western blot analysis or immunostaining we obtained and used antibodies as previously described (Cheng et al., 2003). For immunoblotting or immunostaining, we employed the following: polyclonal rabbit antibody against human AIF (1:1000, 1 mg/ml; Chemicon, Temecula, CA), human p53 (1:1000, Cell Signaling, Beverly, MA), and human NMDAξ1 (NR1, 1:1000, 0.2 mg/ml; Santa Cruz Biotechnology, Santa Cruz, CA) that all crossreact with the corresponding mouse antigens; mouse NR2A (1:500, 1 mg/ml; Upstate Biotechnology, Lake Placid, NY); and mouse *Bax* (1:100, Chemicon). All antibodies against PARs were from Santa Cruz Biotechnology, and various antibodies were used that all crossreact with the corresponding mouse PARs. Polyclonal goat antibodies against N terminus of mouse PAR1 (S-19) and mouse PAR2 (S-19) and against the C terminus of mouse PAR3 (M-20) and mouse PAR4 (M-20) were used as negative controls. Miscellaneous chemical reagents were obtained as described (Cheng et al., 2003). The peptides, Ac-DEVD-CHO (caspase-3) and z-IETD-fmk (caspase-8) were obtained selected on the basis of substrate specificity (Budd et al., 2000) and were from Sigma. Nω-Nitro-L-arginine methyl ester (L-NAME), Nω-Nitrol-L-arginine (N-Arg), and L-arginine were purchased from Sigma. N^{ω}-monomethyl-L-arginine (Me-Arg) was obtained from Calbiochem. NOS inhibitors were applied at 100–500 μM.

Neuronal Cultures

Primary neuronal cultures were established as described (Bonfoco et al., 1995). In brief, cerebral cortex was dissected from fetal C57BL/6J mice or PAR1 null mice (Coughlin, 2000) at 16 days of gestation, treated with trypsin for 10 min at 37°C, and dissociated by trituration. Dissociated cell suspensions were plated at 5×10^5 cells per well on 12-well tissue culture plates or at 4×10^6 cells per dish on 60 mm tissue culture dishes coated with poly-D-lysine, in serum-free Neurobasal medium plus B27 supplement (GIBCO-BRL, Rockville, MA). The medium suppresses glial growth to <2% of the total cell population. The absence of astrocytes was confirmed by the lack of glial fibrillary acidic protein (not shown). Cultures were maintained in a humidified 5% CO_2 incubator at 37°C for 7 days before treatment. Medium was replaced every 3 days.

NMDA-Induced Apoptosis in Neuronal Culture

For induction of neuronal apoptosis, cultures were exposed for 10 min to 300 μM NMDA/5 μM glycine in Mg^{2+}-free Earle's balanced salt solution (EBSS) (Bonfoco et al., 1995). Control cultures were exposed to EBSS alone. After the exposure, cultures were rinsed with EBSS, returned to the original culture medium, and incubated with different concentrations of either human APC (1–100 nM) or recombinant mouse APC (1–100 nM) for 0, 3, 6, 12, 24, and 36 hr, protein C zymogen (100 nM), anti-APC IgG (C3, 11 μg/ml), Ser360-Ala-APC (100 nM), or boiled APC (100 nM) for 24 hr. Ac-DEVD-CHO (50 μM) and z-IETD-fmk (20 μM) were applied 3 hr prior to NMDA treatment. Different anti-PAR antibodies (20 μg /ml) were added to the incubation medium simultaneously with APC after NMDA exposure. In typical inhibition experiments, we used either 100 nM human plasma-derived APC (hAPC) or 10 nM mouse recombinant APC (mAPC). TFLLRNPNDK (10 μM) and SLIGRL (100 μM) were added to the incubation medium after NMDA exposure.

Staurosporine-Induced Apoptosis in Neuronal Culture

Staurosporine (0.5 μM, 0.05% DMSO) was added to the culture medium for 0, 3, 6, 12, 18, 24, and 48 hr. Controls were exposed to 0.05% DMSO. Ac-DEVD-CHO (50 μM) and z-IETD-fmk (20 μM) were applied 3 hr prior to staurosporine treatment.

Detection of Apoptosis

Apoptotic cells were visualized by in situ terminal deoxynucleotidyl transferase-mediated digoxigenin-dUTP nick-end labeling (TUNEL) assay according to manufacturer's instructions (Intergen Company, Purchase, NY). Cells were counterstained with the DNA binding fluorescent dye, Hoechst 33342 (Molecular Probes, Eugene, OR) at 1 mg/ml for 10 min at room temperature to reveal nuclear morphology. The number of apoptotic cells was expressed as the percentage of TUNEL-positive cells of the total number of nuclei determined by Hoechst staining. The cells were counted in 10–20 random fields (30× magnification) by two independent observers blinded to the experimental conditions. The number of apoptotic cells under basal conditions (vehicle only) was subtracted from the number of apoptotic cells in various experimental groups.

In Situ DNA Fragmentation/Caspase-3 Staining

Subsequent to visualization of fragmented DNA with TUNEL, cells were permeabilized with 0.4% Tween 20 for 30 min and blocked with 10% normal goat serum in PBS for 30 min at room temperature. A primary anti-caspase-3 antibody was applied overnight at 4°C. After washing in PBS three times, cells were incubated with Rhodamine-conjugated goat anti-rabbit IgG (1:150) for 1 hr at 37°C.

Caspase-3 and -8 Activity

Cells were washed with PBS and resuspended in cell lysis buffer. 50 μl of lysates was incubated with 50 μM caspase-3 (DEVD-pNA) or caspase-8 (IETD-pNA) substrate at 37°C (ApoAlert caspase assay kit, Clontech, Palo Alto, CA). Substrate hydrolysis was determined as absorbance change at 405 nm in a microplate reader and enzymatic activity was expressed in arbitrary units per mg of protein.

Western Blot Analyses

Whole cellular extracts and nuclear proteins or cell membrane fractions were prepared and protein concentration determined using Bradford protein assays (Bio-Rad, Hercules, CA); 10–50 μg of protein was analyzed by 10% SDS-PAGE and transferred to nitrocellulose membranes that were then blocked with 5% nonfat milk in TBS (100 mM, Tris [pH 8.0], 1.5 M NaCl, 0.1% Tween 20) for 1 hr. The membranes were incubated overnight with primary antibodies in TBS and then washed and incubated with a horseradish peroxidase-conjugated secondary antibody for 1 hr. Immunoreactivity was detected by using the ECL detection system (Amersham, Piscataway, NJ). The relative abundance of each protein was determined by

scanning densitometry, using β-actin as an internal control. Data from multiple Western blots (n = 3–5) were averaged for statistical analysis.

Reverse Transcription-Polymerase Chain Reaction Analysis

Total RNA was isolated from cells and RT-PCR analyses of p53 and GPDH were performed as described (Cheng et al., 2003).

cGMP Assay

Ten minutes after NMDA application with or without NOS inhibitors, the cells were lysated with 0.1 N HCL. cGMP was assayed with an Amersham ^{3}H-labeling assay kit per manufacturer's instructions.

Electrophoretic Mobility Shift Assay

Nuclear proteins were extracted from cortical neuronal cultures at 0, 0.5, 1, 2, 3, and 6 hr after exposure to NMDA using NE-PER nuclear and cytoplasmic extraction reagents according to manufacturer's instructions (Pierce, Rockford, IL). Human umbilical vein endothelial cells (HUVEC) were exposed to lipopolysaccharide (LPS) (*E. coli*, 200 ng/ml) for 4 hr as a positive control. The activation of NF-κB was determined by its binding to the consensus sequence (5′ AGT TGA GGG GAC TTT CCC AGG 3′). Briefly, NF-κB consensus oligonucleotides (Promega, WI) were labeled using digoxigenin gel shift kit (Roche, Indianapolis, IN). Labeled oligonucleotides were incubated with 30 μg nuclear protein extracts at room temperature for 20 min in the reaction buffer (Roche). Nuclear extracts incubated with NF-κB consensus sequence were run immediately on 4% native polyacrylamide gel in 0.25× TBE. The gel was transferred to Nitron$^+$ membrane (Amersham) and the signal detected according to the manufacturer's manual (Roche).

Intrastriatal NMDA Microinjections in Mice

All procedures were done in accordance with protocols approved by the University of Rochester. Male C57BL/6J mice, PAR1 null (Coughlin, 2000), PAR3 null, and PAR4 null mice (Nakanishi-Matsui et al., 2000) weighing 23–25 g were anesthetized with i.p. ketamine (100 mg/kg) and xylazine (10 mg/kg). Animals received micro-infusions into the right striatum (0.5 mm anterior, 2.5 mm lateral, 3.2 mm ventral to the bregma) of either vehicle, NMDA (20 nmol in 0.3 μl of PBS [pH 7.4]), NMDA + recombinant mouse APC (0.002 μg or 0.02 μg or 0.2 μg), NMDA + APC (0.2 μg) + anti-PAR1 (H-111, 0.2 μg) or anti-PAR2 (SAM 11, 0.2 μg) or anti-PAR3 (H-103, 0.2 μg) antibodies. The solutions were infused over 2 min using a microinjection system (World Precision Instruments, Sarasota, FL). The needle was left in place for an additional 8 min after the injection as reported (Ayata et al., 1997). After 48 hr, mice were sacrificed under deep anesthesia for analysis of exitotoxic lesions. Mice were transcardially perfused with PBS (pH 7.4) followed by 4% paraformaldehyde. The brains were removed and coronal sections at a 30 μm thickness were prepared using Vibratome. Every fifth section 1 mm anterior and posterior to the site of injection was stained with cresyl violet. The lesion area was identified by the loss of staining as reported (Ayata et al., 1997). The lesion areas were determined by an image analyzer (Image-ProPlus, Media Cybernetics, Silver Spring, MD) and integrated to obtain the volume of injury.

Statistical Analysis

Data were presented as mean ± SEM. ANOVA was used to determine statistically significant differences. $p < 0.05$ was considered statistically significant.

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